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**Title: Preserved Monocyte-Derived Dendritic Cell Differentiation and Maturation  
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**Abstract**

**Background:** Dendritic cells (DC) are fundamental for the initiation of immune responses and are important players in AIDS immunopathogenesis. Impairment of DC function may result from bystander effects of HIV-1 envelope proteins independently of direct HIV-1 infection. HIV-2 envelope proteins are thought to interact with a broader range of receptors than those of HIV-1, and have been shown to have T-cell immunosuppressive properties mediated by monocytes.

**Objective:** Investigate the effects of HIV-2 envelope on DC differentiation and maturation.

**Methods:** The modulatory properties of HIV-2<sub>ROD</sub> envelope on DC generated from monocytes were assessed using both recombinant protein and whole chemically (aldrithiol-2 treated) inactivated virus. DC phenotype was assessed by flow cytometry and DC function by their ability to stimulate allogeneic T-cells and to produce cytokines.

**Results and Conclusion:** We demonstrate that HIV-2 Env had no effects upon DC differentiation and maturation despite its broad receptor usage and ability to modulate monocyte function. Our data support the possibility that preservation of DC function may contribute to the relatively benign course of HIV-2 disease. Understanding the mechanisms that prevent HIV-2 from impairing DC function may provide new therapeutic targets.

## Concise Communication

### **Title: Preserved Monocyte-Derived Dendritic Cell Differentiation and Maturation in the Presence of HIV-2 Envelope**

### **Running Head: HIV-2 Env Effects on Dendritic Cells**

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**Key words:**

HIV-2; HIV envelope proteins; gp105; HIV immunopathogenesis; Dendritic cells.

## Introduction

Dendritic cell (DC) disturbances are thought to significantly contribute to AIDS pathogenesis (recently reviewed in [1, 2]). In addition to direct human immunodeficiency virus (HIV) type 1 infection of these professional antigen-presenting cells, viral envelope (Env) proteins *per se* are able to induce a significant impairment of DC function [2-7]. The HIV-1 surface glycoprotein, gp 120, is readily shed from the cell surface due to its noncovalent association with the transmembrane Env gp41, exerting modulatory effects on bystander cells through binding to CD4, chemokine receptors or other molecules expressed by DC, such as lectins [8-10]. HIV-1 Env proteins were shown to functionally impair isolated primary DC [2, 3, 11], as well as the *in vitro* development and maturation of monocyte-derived DC [4-7]. HIV-2, which is associated with an attenuated form of HIV/AIDS disease as compared to HIV-1 [12-19], has a broader range of coreceptor usage [20-22] and elicits higher levels of neutralizing antibodies [23], suggesting that its Env has structurally distinct properties. Moreover, the external component of HIV2<sub>ROD</sub> Env, gp105, unlike gp120 of HIV-1, was shown to bind to the CD8 molecule with high affinity [24] and to induce higher levels of beta-chemokines [25]. We have also shown that HIV2<sub>ROD</sub> gp105 induced marked suppression of *in vitro* lymphoproliferation to both recall antigens and anti-CD3 through a monocyte contact-dependent mechanism as well as high levels of TNF- $\alpha$  production [26, 27]. Thus, it is reasonable to hypothesize that HIV2<sub>ROD</sub> gp105 significantly impacts on DC differentiation and maturation *in vitro*.

## Methods

Recombinant Env protein (gp105 from HIV-2<sub>ROD</sub> produced in a baculovirus expression system; EU Programme EVA, MRC, UK) as well as whole chemically inactivated virus were used. HIV-2<sub>ROD</sub> grown on H9 cells was inactivated at 4 °C with 1 mM aldrithiol-2 (AT-2; Sigma-Aldrich, St Louis, MO) and purified and concentrated using a protocol kindly provided by J. Lifson and J. Bess [28]. Inactivation was shown to disrupt the nucleocapsid without changing the conformational and functional integrity of the surface Env glycoproteins [29]. Given that only about 0,1% of circulating virions are infectious, it is believed that the exposure of host cells to inactivated HIV may more accurately mimic the most frequent type of *in vivo* cell-virion interaction [2].

A standard *in vitro* DC differentiation system was used. Briefly, monocytes were magnetically isolated from peripheral blood mononuclear cells (PBMC) of healthy donors' buffy coats (Portuguese Institute of Blood) with CD14 Microbeads (Miltenyi Biotec, Bergish Gladbach, Germany). Immature DC (iDC) were generated by culturing monocytes ( $3 \times 10^6$ ) for 6 days in RPMI 1640 medium (Gibco-Invitrogen, Paisley, UK) containing 10% fetal calf serum (Sigma-Aldrich), penicillin/streptomycin (100U/100mg/ml; Gibco-Invitrogen), glutamine (2mM; Gibco-Invitrogen) plus GM-CSF (50 ng/ml; R&D Systems, Minneapolis, MN) and IL-4 (20 ng/ml; R&D Systems), in the absence or presence of recombinant gp105<sub>ROD</sub> (1 µg/ml) or the whole inactivated HIV-2<sub>ROD</sub> (HIV-2Tx; 300 ng of p26 Ag equivalent/ml) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

DC phenotype was assessed by flow cytometry using a panel of monoclonal antibodies against HLA-DR, CD80, CD86 and CD83 (all from BD Biosciences, San Jose, CA), as well as PD-L1 and PD-L2 (from eBioscience, San Diego, CA), as previously described

[26]. Samples were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest (BD Biosciences) and FlowJo (Tree Star, Ashland, OR).

In order to assess if the presence of gp105<sub>ROD</sub> during DC differentiation from monocytes impaired the subsequent DC maturation, DC were stimulated with lipopolysaccharide (LPS; 100 ng/ml; Sigma-Aldrich) for 48 hours.

DC function was further assessed by measuring cytokine production in culture supernatants by ELISA from eBiosciences as well as by evaluating their allostimulatory capability in a mixed-leukocyte reaction. DC were co-cultured with purified allogeneic T cells ( $1 \times 10^5$  cells; Pan T cell Isolation Kit; Miltenyi Biotec) at different DC: T cell ratio. Proliferation was measured after a 5-day culture by [<sup>3</sup>H]thymidine incorporation (Amersham Pharmacia Biotech, Little Chalfont, UK) after a pulse of 1  $\mu$ Ci during the final 18 h culture, and cpm were counted in a gaseous scintillation  $\beta$ -counter (Packard, Meriden, CT, USA). Results were analyzed as stimulation indexes calculated by dividing the cpm obtained in DC: T cell co-culture by the cpm obtained in T cells cultured alone.

## Results

As shown in Fig. 1A, iDC derived from monocytes in the presence of gp105<sub>ROD</sub> (iDC/gp105) exhibited expression levels of HLA-DR, CD80, CD86 and CD83 similar to those generated in its absence. The iDC generated in the presence of gp105<sub>ROD</sub> were fully competent to undergo subsequent LPS-induced maturation as evaluated phenotypically by flow cytometry (Fig. 1A) and morphologically by optical microscopy (data not shown). As shown in Fig. 1B, DC differentiated in the presence or absence of gp105 showed similar allostimulatory activity after subsequent LPS-induced maturation.



In parallel, similar experiments were performed using whole inactivated virus. As illustrated in Fig. 1E, the treatment with AT-2 fully abrogated viral replication in activated PBMC as documented by the absence of p24 in the supernatants as assessed by ELISA. Microvesicles, prepared from supernatants of uninfected H9 cells using the same procedures used to prepare HIV-2Tx, were used as negative control in all the HIV-2Tx experiments and found to induce no effects (data not shown). Similarly to gp105, the presence of HIV-2Tx during DC differentiation from monocytes did not alter DC phenotype (Fig. 1C), nor did it affect their ability to mature upon LPS stimulation as assessed by the DC morphology (data not shown), expression of co-stimulatory molecules (Fig. 1C) or ability to stimulate allogeneic T cells (Fig. 1D). Programmed death-1 (PD-1) signalling mediates an inhibitory pathway of T-cell responses and its over-expression is currently considered to contribute significantly to the impairment of specific T-cell responses in HIV-1 infected individuals [30]. HIV-1 disease progression is also associated with an up-regulation of PD-L1, a PD-1 ligand [31], and, moreover, HIV-1 derived TLR-7/8 ligands were shown to induce PD-L1 expression on DC and monocytes [32]. Therefore, we also assessed whether the expression of PD-1 ligands, PD-L1 and PD-L2, were up-regulated upon HIV-2Tx exposure. No significant alterations were induced by the presence of the whole inactivated virus (Fig. 1C).

In summary, our data showed that the HIV-2 Env tested does not affect monocyte differentiation into DC.

Next we asked whether HIV-2<sub>ROD</sub> Env *per se* was able to induce DC maturation and if the presence of HIV-2<sub>ROD</sub> Env concomitantly with LPS might modulate this process. For this purpose, iDC were cultured for 48h in the absence or presence of either gp105<sub>ROD</sub> (Fig. 2A and 2B) or HIV-2Tx (Fig. 2C e 2D) with or without LPS, and evaluated with respect to

their morphology (data not shown), phenotype and allostimulatory capacity. As illustrated in Fig. 2, iDC exposed to gp105<sub>ROD</sub> or HIV-2Tx did not exhibit differences in the expression of maturation markers as compared to unexposed iDC. On the other hand, DC maturation induced by LPS was not affected by the presence of either gp105<sub>ROD</sub> or HIV-2Tx during this process (Fig. 2). Thus, we conclude that the HIV-2 Env tested was unable to induce DC maturation *per se*, and, moreover, did not affect LPS-induced DC maturation. Of note, similar findings were obtained using higher concentrations of protein or virus (supplemental figure).

We have previously reported that HIV-2 gp105<sub>ROD</sub> induces high levels of TNF- $\alpha$  production by monocytes [26]. Therefore, we investigated whether DC exposure to either gp105<sub>ROD</sub> or HIV-2Tx could modify their ability to secrete TNF- $\alpha$  or IL-10, an immunosuppressive cytokine shown to be induced by HIV-1 Env [2]. Cytokines were measured by ELISA in supernatants post-48h culture. As shown in Fig. 2E, secretion of both cytokines in the absence or the presence of LPS was not significantly altered by the presence of either gp105<sub>ROD</sub> or HIV-2Tx, as compared to unexposed DC.

## Discussion

To our knowledge, there are no previous studies on the effects of HIV-2 Env on DC differentiation and maturation. We report here no significant alterations induced by the presence of Env recombinant protein or whole chemically inactivated HIV-2<sub>ROD</sub> on DC differentiation from monocytes or in their maturation upon LPS stimulation, as assessed by morphology, expression of co-stimulatory molecules, cytokine production and allostimulatory ability.

Remarkably, the same recombinant protein was previously shown to significantly alter monocyte and T-cell function [26, 27]. Despite some discrepant results according to the strains used, it is widely accepted that HIV-1 Env triggers signalling events that result in abnormal monocyte-derived DC differentiation and maturation *in vitro* and induces significant disturbances of primary DC function [2]. Circulating DC from HIV-1 infected patients exhibit quantitative and qualitative abnormalities [33-36] and Env has been implicated in these impairments [2, 3, 11]. In agreement with a distinct impact of HIV-2 and HIV-1 on DC, it was recently shown that primary DC are less susceptible to HIV-2 than HIV-1 infection *in vitro* using both HIV-2 CCR5 primary isolates and a CXCR4 lab-adapted virus [37]. More HIV-2 strains should be studied to complement our data based on a lab-adapted CXCR4 using strain. Nevertheless, our results suggest that HIV-2 Env may not significantly affect monocyte-derived DC and raise the possibility that myeloid DC may be spared in HIV-2 infection.

HIV-2 is associated with slow rate of AIDS progression and low viremia [15-19, 22, 37-39]. DC play a central role in the generation of immune responses against HIV and opportunistic infections. Moreover, DC may be important in the modulation of HIV-associated immunopathology [1, 2]. Our data raise the possibility that a preservation of DC function may contribute to the relatively benign HIV-2 immunodeficiency. Identification of possible factors that prevent HIV-2 from impairing DC function may significantly increase our understanding of AIDS pathogenesis and provide new therapeutic targets.

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## Figure Legends

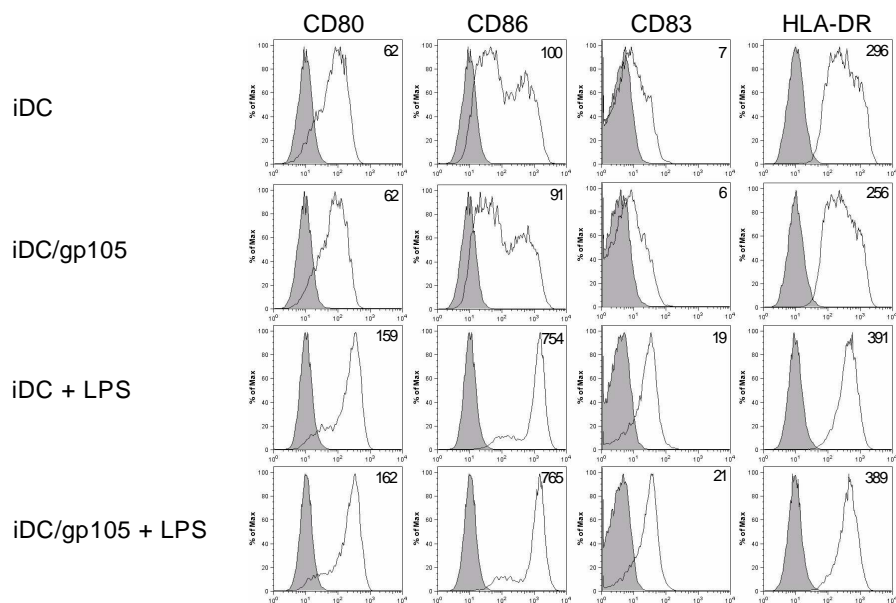
**FIG. 1.** *Effects of HIV-2 Env recombinant protein (gp105<sub>ROD</sub>) and chemically inactivated HIV-2 (HIV-2Tx) on DC differentiation.* iDC were generated from monocytes cultured in medium containing GM-CSF/IL-4 in the absence or presence of gp105<sub>ROD</sub> or HIV-2Tx. After 6 days, cells were cultured with or without LPS for 2 additional days. *Expression of co-stimulatory molecules assessed by flow cytometry cultured in the presence of gp105<sub>ROD</sub> (A) or of HIV-2Tx (C):* numbers in histograms represent the geomean fluorescence intensity of the surface molecules expressed on DC of one representative donor (out of 3). *DC allostimulatory ability of DC generated in the presence of gp105<sub>ROD</sub> (B) or of HIV-2Tx (D):* DC generated in the absence or presence of gp105<sub>ROD</sub> or HIV-2Tx and matured with LPS were co-cultured with purified allogeneic T cells for 5 days; lymphocyte proliferation was measured by [<sup>3</sup>H]thymidine incorporation and stimulation indexes were calculated by dividing the counts per minute (cpm) obtained in the DC: T cell co-cultures by the cpm obtained in the cultures of T cells alone; results are expressed as mean Stimulation Index $\pm$ SEM of 3 donors. (E) Levels of p24 gag protein assessed by ELISA in supernatants collected after 7 day culture of phytohemagglutinin-stimulated PBMC infected with HIV-2Tx or non-inactivated HIV-2 (HIV-2Mock), illustrating the absence of viral replication after AT-2 treatment.

**FIG. 2.** *Impact of gp105<sub>ROD</sub> and HIV-2Tx on DC maturation as well as on DC ability to secrete TNF- $\alpha$  and IL-10.* iDC were generated from monocytes cultured in medium containing GM-CSF/IL-4 in the absence of HIV-2 Env. After 6 days, iDC were treated with gp105<sub>ROD</sub>, HIV-2Tx or with LPS for additional 2 days. In parallel, cells were induced to

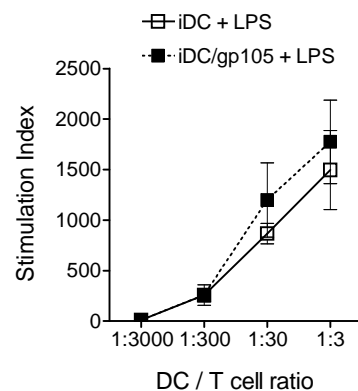
mature with LPS in the presence of gp105<sub>ROD</sub> or HIV-2Tx. DC phenotype was assessed by flow cytometry as illustrated in A (culture with gp105<sub>ROD</sub>) and C (culture with HIV-2Tx). Numbers showed in histograms represent the geomean fluorescence intensity of the surface molecules expressed on DC of one representative donor (out of 3). The DC allostimulatory ability was evaluated after co-culture of DC exposed to gp105<sub>ROD</sub> (B) or to HIV-2Tx (D) with purified allogeneic T cells for 5 days. Lymphocyte proliferation was measured by [<sup>3</sup>H]thymidine incorporation and results are expressed as mean Stimulation Index $\pm$ SEM of 3 donors, calculated by dividing the cpm obtained in the DC: T cell co-cultures by the cpm obtained in the cultures of T cells alone. (E) After 48 hours of culture of iDC with gp105<sub>ROD</sub>, HIV-2Tx with or without LPS, supernatants were collected and IL-10 and TNF- $\alpha$  were measured by ELISA. The graphs show a representative case of the 3 different individuals tested for the effects of gp105<sub>ROD</sub> (top) and a representative case of the 3 different individuals tested for the effects of HIV-2Tx (bottom).

Figure 1

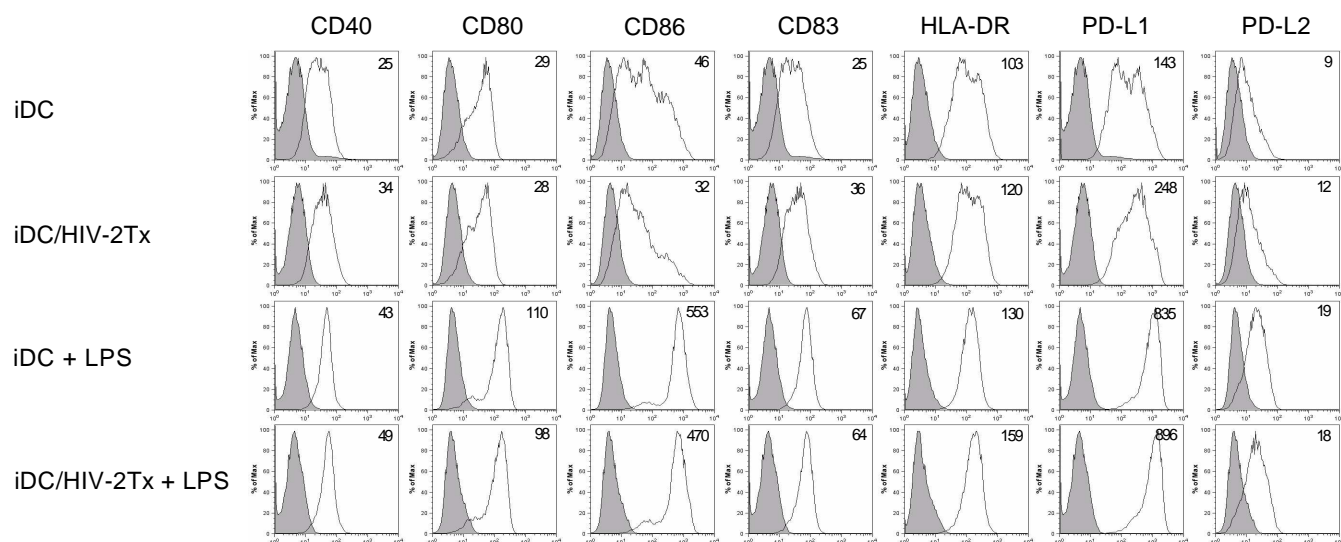
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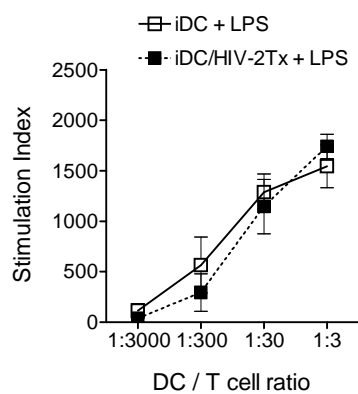
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E

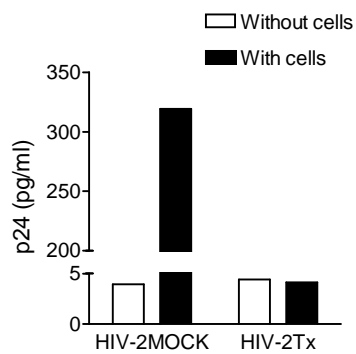
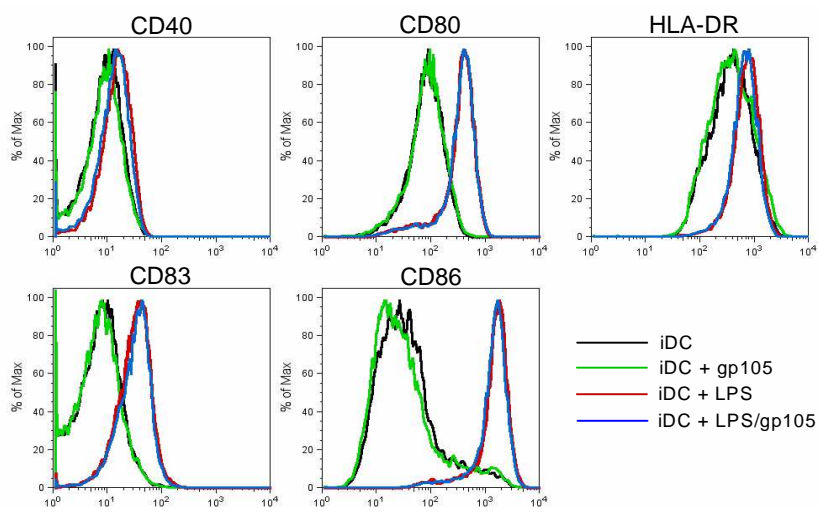
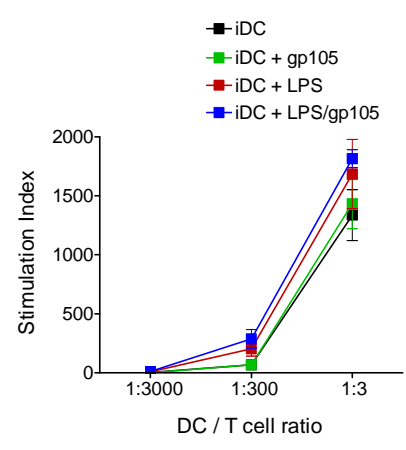


Figure 2

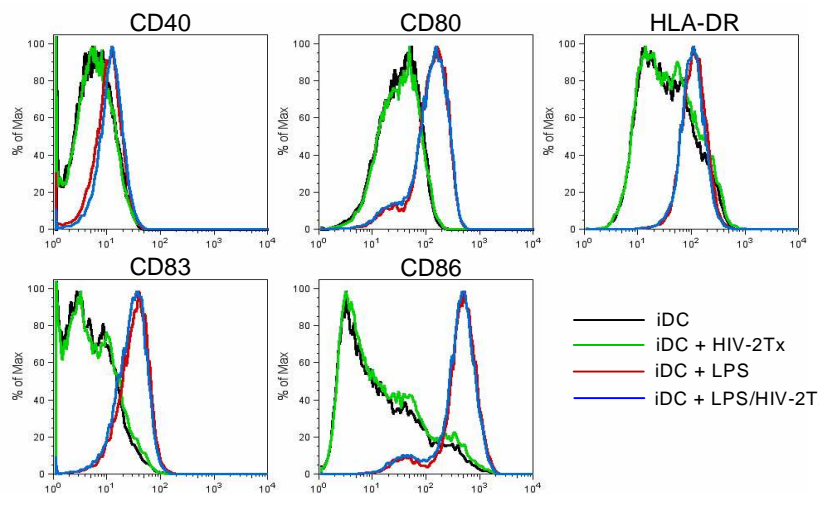
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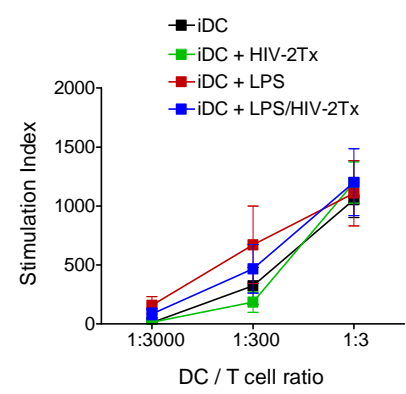
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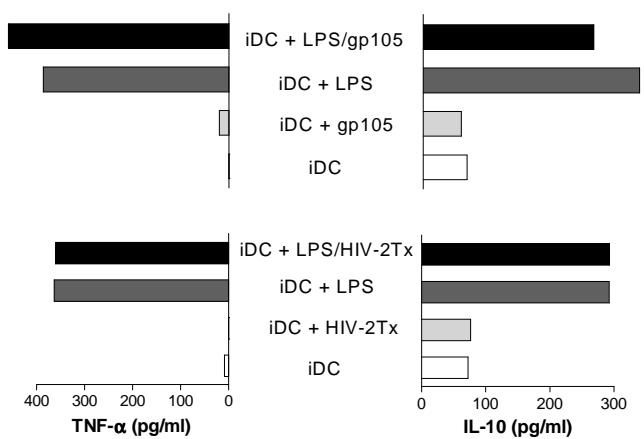
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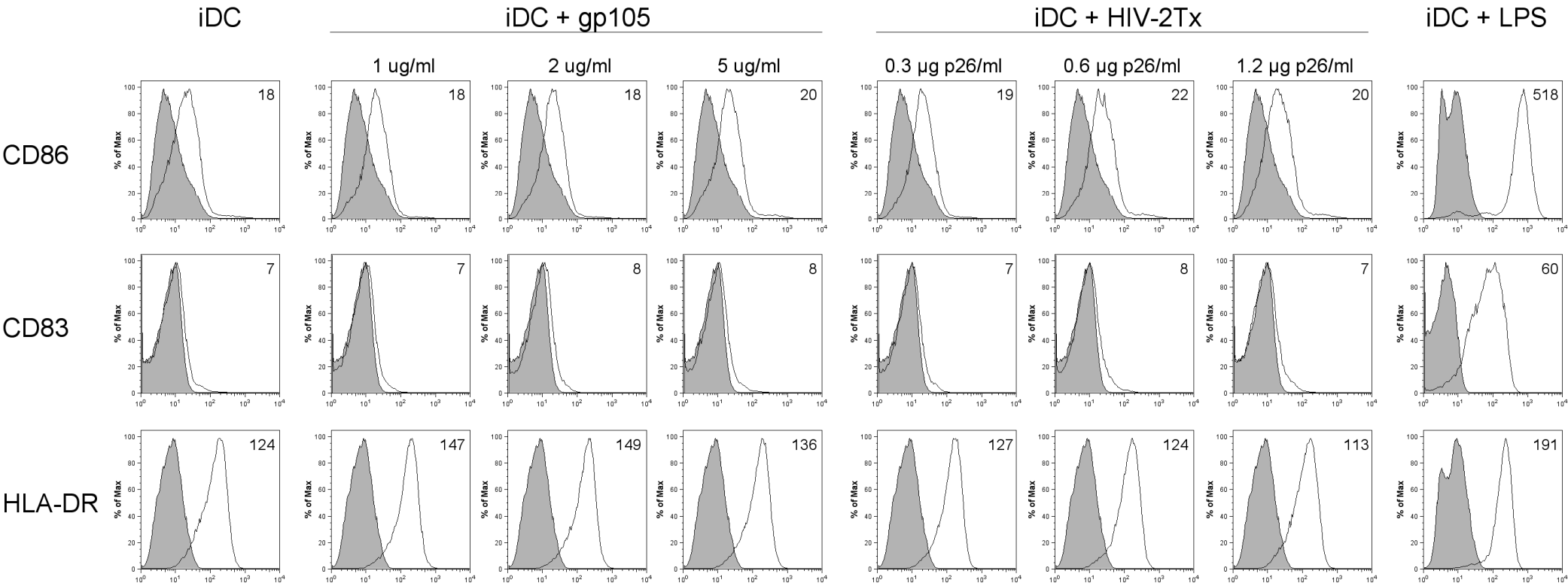


D



E





**Supplemental Figure.** *Dose-dependent effects of gp105ROD or HIV-2Tx on DC maturation.*

iDC were generated from monocytes cultured in medium containing GM-CSF/IL-4. After 6 days, iDC were treated with increasing concentrations of gp105ROD (1, 2 and 5µg/ml) or HIV-2Tx (0.3, 0.6, and 1.2 µg p26/ml) or with LPS (0.1 µg/ml) for additional 2 days. DC phenotype was assessed by flow cytometry as illustrated. Numbers showed in histograms represent the geomean fluorescence intensity of the surface molecules expressed on DC.